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Proportion of DNA Gyrase Mutants among Quinolone-Resistant Strains of *Pseudomonas aeruginosa*

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The proportion of DNA gyrase mutants among quinolone-resistant strains of *Pseudomonas aeruginosa* was examined by introducing the cloned wild-type *Escherichia coli gyrA* and *gyrB* genes. Of 101 spontaneous mutants of *P. aeruginosa* PAO505, 33 (33%) were found to have *gyrA* mutations. Among 17 clinical isolates, 12 (71%) had *gyrA* mutations and 1 (6%) had a *gyrB* mutation.

Quinolone-resistant organisms are increasing as quinolone derivatives are used clinically for various kinds of infections. Quinolone resistance is caused by chromosomal mutations affecting permeability (1, 2, 6–9, 15, 16, 18) or drug susceptibility of DNA gyrase (EC 5.99.1.3) (3–7, 10, 14–20). In *Escherichia coli*, it has been revealed by transformation with cloned wild-type *gyrA* and *gyrB* genes that mutations in the *gyrA* and *gyrB* genes are equally frequent in spontaneous quinolone-resistant mutants, whereas mutations in the former gene predominate in quinolone-resistant clinical isolates (14). In *Pseudomonas aeruginosa*, what kind(s) of mutation predominates is not yet known. Since we found that the cloned wild-type *E. coli gyrA* gene could make a quinolone-resistant *nalA* mutant of *P. aeruginosa* quinolone susceptible just as with a quinolone-resistant *gyrA* mutant of *E. coli*, the proportion of *gyr* mutations in quinolone-resistant *P. aeruginosa* was examined by transformation with cloned *E. coli gyrA* and *gyrB* genes.

P. aeruginosa PAO505 (15), its *nalA* mutant PAO515 (15), and its transport mutant, PAO6002 (*nalB*) (15) and plasmid pME294, which multiplies in *P. aeruginosa* and was constructed by Y. Itoh (11), were used. They were kindly supplied by S. Iyobe. Spontaneous quinolone-resistant mutants of *P. aeruginosa* PAO505 were isolated at mutation frequencies of about 10^{-9} on LB agar (13) containing nalidixic acid at 8 or 16 times the MIC (400 or 800 μg/ml, respectively) or enoxacin at 8 or 12 times the MIC (6.25 or 9.4 μg/ml, respectively). Quinolone-resistant clinical isolates were obtained from patients with urinary or respiratory tract infections. Plasmid pPAW207 carrying the wild-type *E. coli gyrA* gene was constructed by inserting a 4.5-kilobase filled-in *StuI-SplI* fragment containing the *gyrA* gene from pAW011 (20) into the *SmaI* site of pME294 (11). Plasmid pPBW801 carrying the wild-type *E. coli gyrB* gene was made by inserting a 3.6-kilobase *SspI-BalI* fragment containing the *gyrB* gene from pJB11 (19) into the same site of pME294. These plasmids have a carbenicillin resistance gene as a selective marker. The quinolones used were synthesized in our laboratories. Carbenicillin, novobiocin, and chloramphenicol were purchased from Sigma Chemical Co., St. Louis, Mo., and gentamicin was purchased from Shionogi & Co., Osaka, Japan. Transformation was done by the $CaCl_2$ method (12), and transformants were selected on LB agar containing carbenicillin at 200 μg/ml for carbenicillin-susceptible strains and at 3,200 μg/ml for carbenicillin-resistant strains.

The *nalA* mutant PAO515 was 16-fold or more resistant than its parent, PAO505, to nalidixic acid and enoxacin (Table 1). When it was transformed with pPAW207, carbenicillin-resistant transformants appeared at the same frequency as when it was transformed with pME294, and all of the transformants checked were fully quinolone susceptible without segregation, indicating that the wild-type *E. coli gyrA* gene carried on pPAW207 complemented the mutant *P. aeruginosa nalA* gene on the chromosome without recombination and conferred quinolone susceptibility on the cells. This phenomenon was not due to gene dosage effect because the parent strain, PAO505, remained quinolone susceptible when it was transformed with a similar plasmid with the quinolone-resistant *E. coli gyrA* allele (data not shown). This result also shows that the recombination between *E. coli* and *P. aeruginosa gyrA* genes does not cause quinolone resistance under the experimental conditions employed, although PAO505 is not genotypically recombination deficient. These results show that the *nalA* gene of PAO515 corresponds to the *gyrA* gene and that the wild-type *E. coli gyrA* gene is dominant over the quinolone-resistant *gyrA* gene in *P. aeruginosa*. When PAO515 was transformed with pPBW801, it remained quinolone resistant. It was interesting that susceptibility of the pPAW207 transformant to quinolones was similar to that of PAO505 (MICs of nalidixic acid and enoxacin: 50 and 0.78 μg/ml, respectively) and distinct from that of *E. coli* KL16 (MICs of nalidixic acid and enoxacin: 3.13 and 0.1 μg/ml, respectively) from which the wild-type *gyrA* gene was derived.

Spontaneous quinolone-resistant mutants were isolated from PAO505 by nalidixic acid or enoxacin selection. These

TABLE 1. Quinolone resistance of PAO515 (*nalA*) before and after transformation with the plasmid containing the wild-type *E. coli gyrA* or *gyrB* gene

Strain	Relevant genotype	Plasmid	MIC (μg/ml)	
			Nalidixic acid	Enoxacin
PAO505	Wild type		50	0.78
PAO515	*nalA*		>800	12.5
PAO515	*nalA*	pPAW207[a]	50	0.78
PAO515	*nalA*	pPBW801[b]	>800	12.5

[a] pPAW207 contains the wild-type *E. coli gyrA* gene.
[b] pPBW801 contains the wild-type *E. coli gyrB* gene.

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TABLE 2. Resistance of spontaneous quinolone-resistant mutants of *P. aeruginosa* PAO505 to various antibacterial agents[a]

Strain (*n*)	Type (*n*)	MIC (μg/ml)									Mutation
		NA	ENX	NFLX	OFLX	CPFX	CBPC	NB	CP	GM	
PAO505	Wild type	50	0.78	1.56	0.78	0.39	50	200	25	3.13	
PAO515	*nalA*	>800	12.5	12.5	12.5	3.13	50	200	25	3.13	*gyrA*
PAO6002	*nalB*	800	6.25	6.25	6.25	0.78	400	800	200	0.78	*nalB*
PAO505											
NA selected (38)	1 (22)	>800	12.5	12.5	12.5	3.13	50	200	25	3.13	*gyrA*
	2 (16)	≥800	6.25–12.5	6.25	6.25	1.56–3.13	400	800	200	0.78–1.56	*nalB*?
ENX selected (63)	1 (11)	>800	12.5	12.5	12.5	3.13	50	200	25	3.13	*gyrA*
	3 (52)	200	12.5	12.5	12.5	3.13	6.25	200	100–200	0.78–1.56	*nfxB*?

[a] NA, Nalidixic acid; ENX, enoxacin; NFLX, norfloxacin; OFLX, ofloxacin; CPFX, ciprofloxacin; CBPC, carbenicillin; NB, novobiocin; CP, chloramphenicol; GM, gentamicin.

mutants were divided into three types according to their drug resistance patterns (Table 2). Type 1 mutants were resistant to all of the quinolones tested but were susceptible to unrelated antibiotics. Twenty-two (58%) of 38 nalidixic acid-selected mutants and 11 (17%) of 63 enoxacin-selected mutants belonged to this type. All of the type 1 mutants became as susceptible as the parent, PAO505, to nalidixic acid and enoxacin when transformed with pPAW207 but did not change in susceptibility when they were transformed with pPBW801, indicating that their quinolone resistance was due to *gyrA* mutations. Type 2 mutants were resistant not only to quinolones but also to carbenicillin, novobiocin, and chloramphenicol and at the same time were hypersusceptible to gentamicin. This resistance pattern was similar to that of a *nalB* mutant, PAO6002, which is known to be resistant via decreased drug penetration (15). Sixteen (42%) of 38 nalidixic acid-selected mutants but none of enoxacin-selected mutants belonged to this type. Type 3 mutants were resistant to quinolones and chloramphenicol and concurrently hypersusceptible to carbenicillin and gentamicin. Their resistance pattern resembled that of a *nfxB* mutation causing decreased drug transport (7), except for their resistance to chloramphenicol. Fifty-two (83%) of 63 enoxacin-selected mutants and none of nalidixic acid-selected mutants were of this type. Type 2 and 3 mutants remained unchanged in nalidixic acid or enoxacin resistance when transformed with either pPAW207 or pPBW801, suggesting that the mechanism of their quinolone resistance was not due to mutations in the *gyrA* or *gyrB* gene.

Finally, we examined the proportion of *gyr* mutations in 17 quinolone-resistant clinical isolates of *P. aeruginosa* (Table 3). Fourteen strains were derived from urinary tract infections, and three strains were from respiratory tract infections. Two strains (916-2 and 975) were resistant only to quinolones just like type 1 mutants. The other strains were resistant to quinolones and some or all antibiotics tested. There were no strains with the same resistance pattern as that of type 2 or type 3 mutants. Twelve (71%) of 17 strains that were highly or moderately resistant to quinolones became more or less susceptible to quinolones, e.g., enoxacin (MICs, 0.78 to 25 μg/ml), when they were transformed with pPAW207 but remained unchanged in resistance to the quinolones when they were transformed with pPBW801. Therefore, these 12 strains were considered to have mutations in the *gyrA* gene. One strain 1258-1 became eightfold more susceptible to enoxacin when transformed with pPBW801 but not with pPAW207, suggesting that it had a *gyrB* mutation. Some of the *gyr* mutants may have had additional mutations, because they were not as susceptible to enoxacin as PAO505 after transformation. All of the

TABLE 3. Resistance of clinically isolated *P. aeruginosa* strains to various antibacterial agents[a]

Strain[b]	MIC (μg/ml)									Mutation
	NA	ENX	NFLX	OFLX	CPFX	CBPC	NB	CP	GM	
916-2	800	6.25	3.13	3.13	0.78	50	200	25	3.13	*gyrA* (0.78)[c]
975	>800	50	25	25	6.25	50	200	25	3.13	*gyrA* (0.78)
1011-1	>800	50	50	100	6.25	200	800	200	>200	*gyrA* (6.25)
1258-1	>800	50	100	50	12.5	400	400	400	6.25	*gyrB* (6.25)
1280-2	>800	25	25	50	6.25	400	100	25	>200	*gyrA* (0.78)
1327-1	>800	200	200	400	200	400	400	200	12.5	*gyrA* (25)
KP-5	>800	200	200	200	50	400	400	200	50	*gyrA* (3.13)
KP-6	>800	200	200	100	50	>400	400	200	>200	
KP-20	>800	25	25	25	6.25	>400	400	200	3.13	
KP-23	>800	200	200	100	50	>400	400	200	>200	
KP-24	>800	200	200	200	100	>400	800	200	6.25	
KP-25	>800	25	12.5	25	3.13	50	200	100	6.25	*gyrA* (1.56)
KP-26	>800	100	100	200	25	400	400	200	6.25	*gyrA* (1.56)
KP-34	>800	25	25	50	12.5	50	200	100	6.25	*gyrA* (3.13)
TM-18	800	12.5	12.5	12.5	3.13	50	200	100	12.5	*gyrA* (1.56)
TM-28	>800	12.5	12.5	12.5	3.13	25	≤25	12.5	25	*gyrA* (1.56)
TM-58	>800	12.5	12.5	12.5	3.13	12.5	≤25	25	25	*gyrA* (1.56)

[a] See Table 2, footnote *a*.

[b] The strains were obtained from patients with urinary tract infections except for TM-18, TM-28, and TM-58, which were obtained from patients with respiratory tract infections.

[c] Numbers within parentheses are MICs of enoxacin for the transformants.

strains that were concurrently resistant to antibiotics remained resistant to these antibiotics after transformation with pPAW207 or pPBW801 (data not shown). The other four clinical isolates (24%) remained unidentified with respect to *gyr* mutations because their carbenicillin resistance was so high that it prevented transformation.

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